Role of Two Acetylcholine Receptor Subunit Domains in Homomer Formation and Intersubunit Recognition, as Revealed by α3 and α7 Subunit Chimeras†

Miguel García-Guzmán,‡,§ Francisco Sala,‖ Salvador Sala,⊥ Antonio Campos-Caro,‡ and Manuel Criado*,‡

Departments of Neurochemistry, Pharmacology, and Physiology and Instituto de Neurociencias, University of Alicante, Ap.374, 03080-Alicante, Spain

Received August 8, 1994; Revised Manuscript Received September 21, 1994®

ABSTRACT: Differential expression of subunit genes from the nicotinic acetylcholine receptor (AChR) superfamily yields distinct receptor subtypes. As each AChR subtype has a specific subunit composition and many subunit combinations appear not to be expressed, each subunit must contain some information leading to proper assembly. The neuronal AChR subunits α3 and α7 are expressed in bovine chromaffin cells, probably as constituents of two different AChR subtypes. These subunits have different assembly behavior when expressed in heterologous expression systems: α7 subunits are able to produce homomeric AChRs, whereas α3 subunits require other "structural" subunits for functional expression of AChRs. This feature allows the dissection of the requirements for subunit interactions during AChR formation. Analysis of α7/α3 chimeric constructs identified two regions essential to homomeric assembly and intersubunit recognition: an N-terminal extracellular region, controlling the initial association between subunits, and a second domain within a region comprising the first putative transmembrane segment, M1, and the cytoplasmic loop coupling it to the pore-forming segment, M2, involved in the subsequent interaction and stabilization of the oligomeric complex.

Ligand-gated ion channels are oligomeric membrane proteins involved in signal transmission at neuronal synapses [reviewed in Betz (1990), Barnard (1992), Changeux et al. (1992), and Jessel and Kandel (1993)]. Each receptor family is composed of many different subunits, which are expressed in assorted combinations of dissimilar channel properties, thus increasing functional diversity. The neuronal nicotinic acetylcholine receptor (AChR)[1] family is composed of agonist-binding (α2, α3, α4, and α5) and structural (β2, β3, and β4) subunits, which combine to form pentameric heterooligomers. Until the 1990's, all functional AChRs were reported to contain both subunit types [reviewed in Sargent (1993), and Papke (1993)]. However, two new members of this family (α7 and α8 subunits) have been described, which differ in their association characteristics (Schoepfer et al., 1990; Couturier et al., 1990; Séguéla et al., 1993; Peng et al., 1994). In heterologous expression systems, α7 and α8 subunits can form homomeric AChRs and seem unable to assemble with other members of the family (Couturier et al., 1990; Séguéla et al., 1993; Gerzanich et al., 1994). Immunoprecipitation studies also indicate that α7 subunits are not associated in vivo with previously reported subunits (Vernallis et al., 1993), although it has been suggested that they may associate with other undiscovered subunits (Anand et al., 1993). This different assembly behavior may reflect the existence of specific recognition domains in each subunit type and could be used to establish a model system in which to study the regions involved in intersubunit recognition. Recently, we have cloned the bovine α3 (Criado et al., 1992) and α7 (García-Guzmán et al., 1995) subunits of neuronal nicotinic AChRs, which are probably present in chromaffin cells as components of two different AChR subtypes. We have now exploited the aforementioned differences between α3 and α7 subunits in multimer assembly by constructing α3/α7 chimeras and examining (a) the ability of the *Xenopus* oocyte expression system to produce functional homomeric AChRs when chimeras were expressed separately and (b) the production of functional AChRs when chimeras were coexpressed in pairwise combinations. This approach allowed us to reinforce and extend previous reports indicating the importance of the N-terminal domain of subunits in intersubunit recognition (Yu & Hall, 1991; Verrall & Hall, 1992; Sumikawa & Gehle, 1992; Chávez et al., 1992; Hall, 1992) and homo-oligomerization (Kuhse et al., 1993) and to demonstrate the importance of a short domain of 37 amino acids which includes the first putative transmembrane segment, M1, and seems essential for homomer formation.

MATERIALS AND METHODS

Construction of Chimeras. When possible, chimeric assembly was performed using restriction sites common to both subunits (*Sac*I and *Nar*I, see Figure 1, α7 subunit). Other chimeras and point mutations were made, essentially as described previously (Herlitze & Koenen, 1990), by performing two successive PCR amplifications. In the first PCR (20 cycles: 94 °C for 1 min; 48 °C for 1 min; 72 °C

† This work was supported by Research Grants PB92-0346 from the Ministry of Education of Spain (DGICYT) and SC1*CT91-0666 from the Commission of the European Economic Community (to M.C.). M.G.-G. was the recipient of a predoctoral fellowship from the Ministry of Education of Spain. Appreciation is also extended to the Alexander von Humboldt Foundation for the donation of a DNA synthesizer.

* Corresponding author.

‡ Department of Neurochemistry.

§ Present address: Max-Planck-Institut für exp. Medizin, D-37075 Göttingen, Germany.

‖ Department of Pharmacology.

⊥ Department of Physiology

® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

[1] Abbreviations: AChR, nicotinic acetylcholine receptor; α-Bgt, α-bungarotoxin; NFR, normal frog Ringer solution.

0006-2960/94/0433-15198$04.50/0

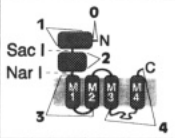
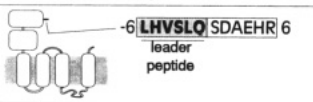
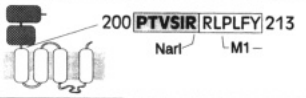
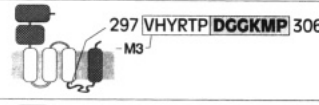
Construct Name	Structure	α-Bgt binding (%)	Current (%)
α7		100±10	100±17
C1		1.2±0.2	0
C2		2.7±0.6	0
C3		13.4±1.7	5.1±1.6
C4		11.0±0.8	3.3±1.1
C5		149±20	0
C6		3.2±0.8	0
C7		2.3±0.3	88.3±15.1

FIGURE 1: Schematic diagrams and functional expression of α3/α7 chimeras. The structure of each chimera is displayed with shaded and open boxes representing α7 and α3 domains, respectively. The diagram of the α7 subunit (top) shows the different domains (1−4) that were considered in this study and the two restriction sites (*Sac*I and *Nar*I) used for some chimeric constructions. The amino acid sequences where the α3 and α7 domains are connected are displayed with some significant features to aid localization. Again, α3 and α7 sequences are indicated by open and shaded boxes, respectively. Amino acid numbering relates to the corresponding subunit. Expression of the different constructs was tested by α-Bgt binding and nicotine-evoked ionic currents. All data were normalized to those obtained with the α7 subunit alone, and means ± SEs of values obtained from at least 24 different oocytes from three different donors are shown. Typical values obtained with the α7 subunit were 3−5 fmol of bound α-Bgt/oocyte and 2−4 μA/oocyte at −40 mV.

for 1 min), 50 ng of target DNA was amplified using a flanking primer and a mutated or chimeric primer (approximately 36 nucleotides in length, about half corresponding to each subunit). The second PCR was carried out with 125 ng of the first PCR product and 1/15th of the product concentration (in molar terms) of the DNA to be mutated or used for chimera construction. Prior to performance of the second PCR, the reaction mixture, without primers, was successively incubated at 94 °C for 5 min (denaturation), 50 °C for 5 min (annealing), and 72 °C for 3 min (extension). Next, the primers were added and PCR was performed (20 cycles: 94 °C for 1 min; 55−60 °C for 1 min; 72 °C for 1.5 min). The mutated or chimeric DNA obtained from the second PCR was sequenced (Sanger et al., 1977) before being manipulated further, with restriction enzymes, for insertion into its appropriate location in the original gene. A brief description of the construction of each chimera follows (see Figure 1): Chimera C1 consists of the α3 sequence preceded by the leader peptide and a short 5′ noncoding region from the α7 subunit. Constructs C2, C3, C6, and C7 were obtained using a *Sac*I site common to the α3 and α7 sequences or a *Nar*I site present in the α3 subunit and introduced, by PCR, as a silent mutation of the α7 subunit. Chimeras C4 and C5 were constructed by introducing, as PCR product, domain 3 or 4 of the α7 subunit into the α3 subunit and then exchanging (by using the common *Nar*I site) transmembrane fragments M1−M4 and the large cytoplasmic loop with the ones present at C3.

Oocyte Expression. Chimeric DNAs were inserted into the pSP64T vector (Krieg & Melton, 1984). Capped mRNA was synthesized in vitro using SP6 RNA polymerase (Boehringer Mannheim). *Xenopus* (Nasco) oocytes were inoculated with 25 ng of RNA in 50 nL of sterile water. Injection of larger amounts of RNA did not yield increasing expression of any of the tested constructs. In co-injection experiments the same amount (25 ng of RNA/50 nL) of each species was injected. Preliminary experiments, performed using 5:1 and 1:1 ratios (chimeric subunit to α7 subunit), yielded the same results, and therefore a 1:1 ratio was used in further experiments. Characterization of the resulting AChRs was performed between 3 and 7 days after injection.

Total surface expression of α-bungarotoxin (α-Bgt)-binding AChRs was tested. Briefly, oocytes were preincubated at 18 °C for 30 min in Barth's solution containing 5% fetal calf serum; then 5 nM [^{125}I]α-Bgt (Amersham) was added, and the oocytes were incubated for 2 h at 18 °C. The excess toxin was removed with five 4-mL washes of Barth's solution. Nonspecific binding was determined using non-inoculated oocytes. Inoculated oocytes, which were preincubated with cold toxin, yielded similar results.

Oocyte membrane currents were measured 3−7 days after RNA injections with a two-microelectrode voltage clamp amplifier. The recording chamber was continuously perfused by gravity (8 mL/min) with normal frog Ringer solution (NFR), and solutions containing different concentrations of nicotinic agonists were applied through a 1.5 mm diameter pipette located close to the oocyte. The composition of NFR was 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes, pH 7.2. Voltage pulses and data acquisition were controlled using a DigiData interface and pCLAMP software (Axon Instruments, Foster City, CA). Currents were filtered at 20 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA), digitized at 250 Hz, and stored on hard disk for later analysis.

RESULTS AND DISCUSSION

Expression of Homomeric AChRs by α3/α7 Chimeras. Coexpression of homologous receptor subunits in an individual cell indicates the existence of predetermined molecular mechanisms controlling the specific identification and interaction of the subunits which regulates the assembly of different receptor subtypes. As an initial approach to determine the contribution of specific subunit domains to intersubunit recognition, we have studied the expression of chimeric proteins composed of two neuronal AChR α subunits, which are characterized by having different assembly requirements. Thus, in the first part of this work we have analyzed the functional expression of homomeric AChRs assembled by the different α3/α7 chimeras (see

Figure 1 for the α7 subunit and the α3/α7 domains in each construct). All chimeras studied were preceded by the leader peptide and the short 5′ noncoding region from the α7 subunit, and so any effect this region had was the same for all constructions. We thus considered the signal peptide of the α7 subunit to be a separate domain (domain 0). Domain 1 consisted of the first 126 amino acids, terminating just before a disulfide loop, which is conserved in all reported nicotinic AChRs and, indeed, most members of the ligand-gated ion channel superfamily. Domain 2 contains the disulfide loop and the major components of the ligand-binding sites, and extends up to, but does not include, the first putative transmembrane segment, M1. Domain 3 includes the hydrophobic segment, M1, and the hydrophilic intracellular loop connecting it with transmembrane segment M2. Finally, the C-terminal region, the hydrophobic segment M4, and the large variable (M3−M4) intracellular loop were collectively termed domain 4. Assembly of AChR homomers was monitored in two ways: by measuring α-bungarotoxin (α-Bgt) binding to the external surface of oocytes, [although this approach was limited to constructs containing domain 2 of the α7 subunit, as this region contains most of the α-Bgt binding determinants (the α3 subunit does not carry α-Bgt binding sites)] and by recording the ionic currents evoked using cholinergic agonists. These approaches are expected to detect only properly assembled AChRs that have followed an accurate maturation pathway from the endoplasmic reticulum to the plasma membrane. Misfolded or unassembled proteins are intracellularly retained and degraded (Hurtley & Helenius, 1989; Blount & Merlie, 1990; Klausner & Sitia, 1990). It is important to note that we used α7 AChR expression as an internal control in all experiments because of the inherent variability of the oocyte expression system: the results are expressed as a percentage of the α7 response.

Previous reports suggest that the hydrophilic N-terminal region of ligand-gated receptors is responsible for intersubunit recognition (Yu & Hall, 1991; Verral & Hall, 1992; Sumikawa & Gehle, 1992; Chávez et al., 1992) and homomer formation (Kuhse et al., 1993); thus we began our analysis with the α3 subunit linked to the signal peptide of the α7 subunit (construct C1) and then extended the α7 sequence, by successively adding domains 1 and 2 (constructs C2 and C3, respectively). Oocytes inoculated with C1 and C2 cRNAs remained silent upon cholinergic stimulation, indicating that the corresponding polypeptides were not able to assemble functional homomeric AChRs. Extension of the α7 sequence to domain 2 (construct C3) yielded detectable AChR expression; however, ionotropic responses and superficial α-Bgt binding levels were much lower than for native α7 AChRs (Figure 1). The C3 chimeras and the intact α7 AChRs were shown to have similar affinities for nicotine and α-Bgt by using a range of different ligand concentrations (data not shown); thus the lower expression levels observed for the C3 construct had to be due to reduced oligomer assembly efficiency, suggesting that other domains are required for α7 homooligomerization.

The putative transmembrane domains 1−3 of α3 and α7 subunits contain amino acid sequences which are highly conserved; therefore the nonconserved structures present in domain 4 of the α7 subunit might be expected to contribute to efficient homomeric assembly. Chimera C4 contains domain 4 from α7 in addition to the α7 domains 1 and 2 present in C3. Compared to construct C3, C4 showed neither improved assembly efficiency nor altered agonist/antagonist affinities and responses (Figure 1). Thus, despite high homology, the region containing the hydrophobic segments M1, M2, and M3 and the corresponding hydrophilic loops connecting them seemed to include specific motifs involved in α7 homomeric folding and assembly.

Construct C5 divides this core region: it was similar to C3, but extended the α7 sequence over domains 1, 2, and 3, i.e., from the N-terminus to the start of the M2 segment. Oocytes inoculated with C5 cRNA actually expressed levels of surface α-Bgt binding sites even higher than that observed for α7 homomers, but no inward currents were detected upon cholinergic stimulation (Figure 1). This observation does not reflect any modification of agonist affinity, as determined by use of a range of agonist concentrations (data not shown), but rather some sort of block or alteration in the mechanism coupling agonist binding with channel gating, as has been suggested for several nicotinic−serotonergic chimeric subunits (Eiselé et al., 1993). Interestingly, sucrose gradient centrifugation of the C5 AChR present in the external membrane of the oocytes revealed a unique molecular species with a size close to that of a *Torpedo* AChR monomer (data not shown). The correct assembly of C5-containing AChRs clearly shows domain 3 as a crucial structural motif controlling α7 subunit homooligomerization.

The approach described above of sequentially adding α7 subunit domains from the N- to the C-terminus of α3 in fact reflected a bias inasmuch as previous reports indicated that the oligomerization domains are present in the N-terminal regions (Yu & Hall, 1991; Verral & Hall, 1992; Sumikawa & Gehle, 1992; Chávez et al., 1992; Kuhse et al., 1993). This assumption imposed certain limitations on the analysis: the aforementioned results demonstrate that domain 3 is necessary for efficient assembly (C5) and indicate that regions within domains 1 and 2 also contribute (C3, C4), but the importance of domains 1 and 2 is unclear. Are domains 1 and 2 necessary, in addition to domain 3, for efficient assembly? To answer this question, we prepared chimeras C6 and C7, in which domains 1 and 2, respectively, of the α7 subunit were replaced by the corresponding α3 regions. Oocytes inoculated with C6 cRNA were silent upon application of different nicotine concentrations, and no α-Bgt binding could be detected at their surface (Figure 1). In contrast, expression of the C7 construct yielded nicotine-sensitive inward currents as large as those of the α7 AChRs (Figures 1 and 3). Currents were not blocked by 100 nM α-Bgt (Figure 3), and toxin binding sites were not detected at the oocyte surface, but this was expected, because the main α-Bgt binding region (within domain 2 of the α7 subunit but not present in the α3 subunit) was absent in the C7 chimera. These results thus identified two domains executing a crucial function in homomeric assembly: the first half of the N-terminal region (domain 1) and the area around the first hydrophobic transmembrane segment, M1 (domain 3). Moreover, the lack of expression of chimeras C2 and C6 is evidence that the two domains are interdependent, and only when both regions were present in the same construct (chimeras C5 and C7) could homooligomeric assembly take place.

Analysis of the Heteromeric Association between Chimeras by Dominant Negative Assays. The existence of chimeras expressing no functional AChRs (C1, C2, and C6) or very

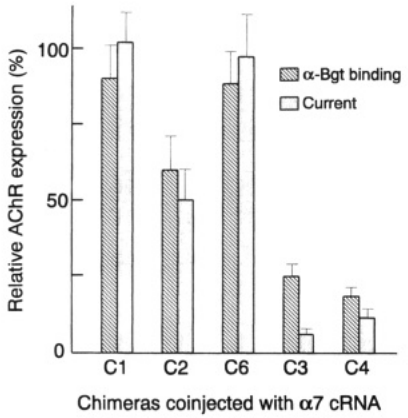


FIGURE 2: Inhibition of α7 AChR surface expression by α3/α7 chimeric subunits. The relative α7 AChR expression in oocytes injected with α7 and chimeric construct cRNAs in a 1:1 ratio is displayed. Data were normalized to those obtained with the α7 subunit alone (100%), and means ± SEs of at least three different experiments (8 oocytes/experiment) are shown.

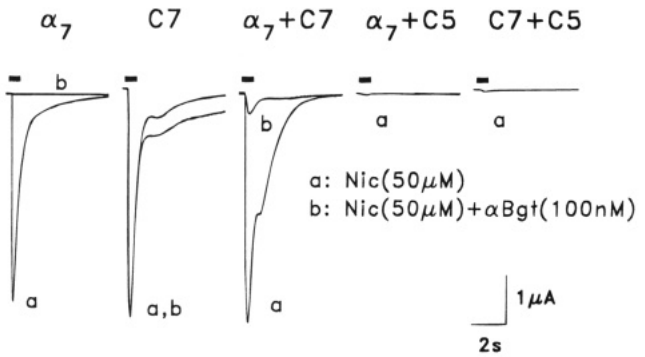


FIGURE 3: Nicotine-activated currents recorded in oocytes expressing α7, C5, or C7 alone or in pairwise combinations. Oocytes were injected with cRNA corresponding to the indicated constructs, either alone or in pairwise combinations. Three to five days later they were challenged with nicotine (50 μM) over 400 ms (horizontal bar). After the responses to nicotine were recorded, oocytes were incubated with α-Bgt (100 nM) for 30 min before being re-exposed to nicotine. The holding potential was −40 mV.

low levels of them (C3 and C4) enabled the performance of negative dominant assays. Verrall and Hall (1992) reported similar assays in which an elegant experimental approach was developed. Truncated mouse muscle AChR subunits were coexpressed with the four wild-type subunits in transfected COS cells. Subunit fragments consisting of the extracellular N-terminal domain alone blocked surface AChR expression by combining with wild-type subunits and, presumably, following a degradation pathway in the endoplasmic reticulum. In our case, by the use of whole and not truncated subunits and simultaneous monitoring of α-Bgt binding and ionic current responses, a new perpective is gained which complements the aforementioned results (Verrall & Hall, 1992). Thus, pairwise co-injection of α7 subunits with the chimeras was performed, and α7 AChR expression at the oocyte surface was monitored as described above. Co-injection of the α7 subunit with C1, an α3 subunit linked to the α7 leader peptide (Figure 2), did not inhibit α7 AChR expression. However, a substantial decrease (50−60%, Figure 2) in α7 AChR expression was observed when coexpressed with C2 constructs. This suggests that the α7 subunit and C2 interact via domain 1, the only region common to both polypeptides, thus emphasizing the importance of this domain for subunit recognition. As mentioned above (Figure 1), an α7 subunit lacking this domain (chimera C6) is unable to form AChR homomers. Furthermore, in co-injection experiments C6 did not influence α7 AChR formation (Figure 2), demonstrating that only domain 1, and not domains 2 and 3, controls the initial α7 association leading to oligomeric assembly. Co-injection of construct C3 inhibited α7 expression even more than C2 co-injection (Figure 2), suggesting that domain 2 contributes to stabilizing the oligomeric complex but only when domain 1 is present in the chimeric construction (i.e., C6 does not interact with α7). The co-injection of α7 with C4 produced similar results to C3 co-injection (Figure 2), demonstrating that domain 4 does not play a role in intersubunit recognition or in the stabilization of α7 specific intermediate folded complexes. These results suggest that domain 1 contains amino acid regions controlling the initial recognition steps between monomeric subunits. Several reports that have analyzed the assembly of other ligand-gated receptors support the idea that regions equivalent to domain 1 control intersubunit association and homomeric assembly. For instance, in the ϵ subunit of the muscle AChR, two amino acids (106 and 115), which would be positioned in the second half of our domain 1, guide AChR assembly in transient transfection experiments (Gu et al., 1991). Additionally, recent studies using chimeric glycine receptor subunits have shown that certain amino acids located in areas equivalent to the second half of our domain 1 are critical for homomeric assembly (Kuhse et al., 1993).

Formation of Hybrid AChRs between α7, C5, and C7 constructs. Expression of either C5 or C7 constructs (Figure 1) yields AChRs that can reach the membrane, and therefore, they would not be expected to produce the kind of dominant negative effects observed with the other chimeras. Thus, we must consider the co-injection of these constructs separately. Three co-injection experiments were performed: (a) α7 subunits with C7 constructs, (b) C5 constructs with C7 constructs, and (c) α7 subunits with C5 constructs. The question addressed by these experiments was whether the co-injected species formed homomeric AChRs independently of each other, or whether they could associate in heteromultimeric AChRs. The latter would indicate recognition between domains from different constructs.

Co-injection of α7 and C7 produced functional AChRs (Figure 3), where the ionic current response was almost totally (92 ± 2%, n=14) inhibited by α-Bgt (Figure 3). As injection of C7 alone produces AChRs which are insensitive to the toxin, the C7 and α7 subunits must be combining to form an α-Bgt-sensitive heteromeric AChR (otherwise, the toxin would have blocked only around 50% of the current, corresponding to independent homomeric α7 AChRs). This reinforces the conclusion that domain 2, which is different in the two species, is not involved in the initial specific recognition process and does not determine a different folding pathway for α3 and α7 subunits. This does not mean that domain 2 cannot mediate the stabilization of the oligomeric complex, and indeed, the presence of domain 2 of the α7 subunit (C3) clearly contributes to the oligomerization process; consider, for example, homomer formation by C3 (Figure 1) and the prominent inhibitory effect of this chimera in the dominant negative assays (Figure 2). Previous findings have located structural domains important for mouse AChR subunit recognition in a region similar to domain 2 (Verral & Hall, 1992). That study describes how a truncated δ

subunit, comprising the first 115 amino acids, did not inhibit subunit heterodimer formation, whereas another reaching amino acid 221 did. Thus, domain(s) between amino acids 115 and 221 appeared responsible for the observed effects. Our domain 1 ends at the position equivalent to δ128 and so overlaps the δ subunit by 13 amino acids. Additionally, one of the amino acid boxes (box 8) apparently mediating specific recognition and association of glycine receptor subunits is also located in this overlap (Kuhse et al., 1993). We suggest that this overlap region contains important determinants for receptor subunit assembly. Interestingly, it has been proposed that a portion of this overlap forms part of subunit interfaces in the mouse muscle AChR (Sine, 1993).

The co-injection of C5 and C7 would have produced functional AChRs if the two constructs had not recognized each other, because C7 can independently do so. However, although α-Bgt binding sites were detected, only small currents were observed (Figure 3). Therefore, heteromeric AChRs must have been formed that were able to bind α-Bgt, as C5 contains the α-Bgt binding determinants, but were unable to allow current flow, because the C5 component caused the formation of silent AChRs. This again indicates that recognition between subunits did not require homology in domain 2. Clearly, hybrid AChRs containing C5 subunits are silent channels regardless of the other contributing subunits, because co-injection of C5 and α7 subunits leads to the same situation as described above: AChRs able to bind α-Bgt but unable to allow current flow (Figure 3). Interestingly, the efficiency of hybrid AChR assembly between C5 and α7, or C5 and C7, as measured by α-Bgt binding sites present in the oocyte membrane was comparable to that obtained when the different constructs (α7 and C5) were separately expressed. This indicates not only that domains 1 and 3, which are common to all three species, are governing oligomeric assembly of the α7 subunit but also that domains 2 and 4, and transmembrane segments M2 and M3, which are different between C5, C7, and α7 constructs, are structurally compatible between α7 and α3 subunits. If they had been incompatible, a decrease in the expression level of hybrid AChRs would have been observed.

Given that domain 1 seems to be controlling the initial recognition process in α7 subunits, we propose that the role of domain 3 is probably related to the stabilization of some critical structural intermediates leading to homomeric assembly. This characteristic is strongly suggested by the experiment in which the capabilities of C2, C3, and C5 chimeras to produce homomeric assembly were compared (Figure 1). Furthermore, the heteromeric assembly of α7, C5, and C7 constructs (Figure 3) reinforces the proposition that this is the role of domain 3. It is interesting to note that in the aforementioned dominant negative assays (Verrall & Hall, 1992) truncated α, γ, and δ subunits having the M1 segment in their sequences were more effective at inhibiting AChR expression than their counterparts which ended just before that segment. The authors' rationale for this related to the presence or absence of a transmembrane anchor in the truncated subunits, rather than to a direct effect of transmembrane segment M1. However, all our constructs were directly comparable, because they were whole subunits. Thus, the dramatic difference in homomer formation between a chimera having the α3 transmembrane segment M1 (C3) and another having the corresponding α7 segment (C5) demonstrates the important role played by this domain in homomer formation, and this may be a better explanation for the increased inhibition observed with the M1-containing truncated subunits (Verrall & Hall, 1992). The proposed role of the region around the first transmembrane domain could have a more general relevance. Recent results obtained with voltage-dependent potassium channels indicate that, in addition to determinants located in the N-terminal region (Li et al., 1992; Shen et al., 1993), the first transmembrane segment, S1, plays an important role in the coassembly of homo- and heterotetrameric potassium channels (Babila et al., 1994). Similarly, a transmembrane domain seems to be crucial in the oligomerization of another membrane protein, the T cell receptor (Cosson & Bonifacino, 1992).

Finally, it might be of interest to remark that the functional modularity observed in other proteins is also present in ligand-gated ion channels, inasmuch as large regions from different subunits can be exchanged without abolishing functional expression, and indeed α-Bgt binding and/or ligand-evoked ionic current can also be retained. This modularity has been even more dramatically demonstrated using $\alpha 7/5HT_3$ chimeric receptors (Eiselé et al., 1993). Therefore, it is tempting to postulate discrete structural modules within domain 1 which could allosterically initiate a stepwise folding process involving other N-terminal regions (domain 2) and the first transmembrane segment (domain 3), finally resulting in a determined quaternary structure. It has been proposed (Green & Claudio, 1993) that intersubunit recognition and association is cumulative, i.e., that subunits are added one at a time, and this would almost certainly involve progressive conformational changes. It is hard to imagine such refolding occurring without considerable communication between these regions responsible for subunit discrimination and association.

ACKNOWLEDGMENT

We thank Dr. F. I. Smillie for his excellent critical review of the manuscript. We also thank our lab colleagues for their help during the performance of these experiments.

REFERENCES

Anand, R., Peng, X., & Lindstrom, J. (1993) *FEBS Lett. 327*, 241−246.

Babila, T., Moscucci, A., Wang, H., Weaver, F. E., & Koren, G. (1994) *Neuron 12*, 615−626.

Barnard, E. A. (1992) *Trends Biochem. Sci. 17*, 368−374.

Betz, H. (1990) *Neuron 5*, 383−392.

Blount, P., & Merlie, J. P. (1990) *J. Cell Biol. 111*, 2613−2622.

Changeux, J.-P., Galzi, J.-L., Devillers-Thiéry, A., & Bertrand, D. (1992) *Q. Rev. Biophys. 25*, 395−432.

Chávez, R. A., Maloof, J., Beeson, D., Newson-Davis, J., & Hall, Z. W. (1992) *J. Biol. Chem. 267*, 23028−23034.

Cosson, P., & Bonifacino, J. S. (1992) *Science 258*, 659−662.

Couturier, S., Bertrand, D., Matter, J.-M., Hernández, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T., & Ballivet, M. (1990) *Neuron 5*, 847−856.

Criado, M., Alamo, L., & Navarro, A. (1992) *Neurochem. Res. 17*, 281−287.

Eiselé, J. L., Bertrand, S., Galzi, J.-L., Devillers-Thiéry, A., Changeux, J. P., & Bertrand, D. (1993) *Nature 366*, 479−483.

García-Guzmán, M., Sala, F., Sala, S., Campos-Caro, A., Stühmer, W., Gutiérrez, L. M., & Criado, M. (1995) *Eur. J. Neurosci.* (in press).
Gerzanich, V., Anand, R., & Lindstrom J. (1994) *Mol. Pharmacol. 45*, 212−220.
Green, W. N., & Claudio, T. (1993) *Cell 74*, 57−69.
Gu, Y., Camacho, P., Gardner, P., & Hall, Z. W. (1991) *Neuron 6*, 879−887.
Hall, Z. W. (1992) *Trends Cell Biol. 2*, 66−68.
Herlitze, S., & Koenen, M. (1990) *Gene 91*, 143−147.
Hurtley, S. M., & Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277−307.
Jessell, T. M., & Kandel, E. R. (1993) *Cell 72*, 1−30.
Klausner, R. D., & Sitia, R. (1990) *Cell 62*, 611−614.
Krieg, P. A., & Melton, D. A. (1984) *Nucleic Acids Res. 12*, 7057−7070.
Kuhse, J., Magalei, D., & Betz, H. (1993) *Neuron 11*, 1049−1056.
Li, M., Jan, Y. N., & Jan, L. Y. (1992) *Science 257*, 1225−1230.
Papke, R. L. (1993) *Prog. Neurobiol. 41*, 509−531.
Peng, X., Katz, M., Gerzanich, V., Anand, R., & Lindstrom, J. (1994) *Mol. Pharmacol. 45*, 546−554.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5468.
Sargent, P. B. (1993) *Annu. Rev. Neurosci. 16*, 403−443.
Schoepfer, R., Conroy, W., Whiting, P., Gore, M., & Lindstrom, J. (1990) *Neuron 5*, 35−48.
Séguéla, P., Wadiche, J., Dineley-Miller, K., Dani, J. A., & Patrick, J. W. (1993) *J. Neurosci. 13*, 596−604.
Shen, N. V., Chen, X., Boyer, M. M., & Pfaffinger, P. J. (1993) *Neuron 11*, 67−76.
Sine, S. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9436−9440.
Sumikawa, K., & Gehle, V. M. (1992) *J. Biol. Chem. 267*, 6286−6290.
Vernallis, A. B., Conroy, W. G., & Berg, D. K. (1993) *Neuron 10*, 451−464.
Verrall, S., & Hall, Z. W. (1992) *Cell 68*, 23−31.
Yu, X. M., & Hall, Z. W. (1991) *Nature 352*, 64−67.